CURRENT MICROBIOLOGY Vol. 25 (1992), pp. 119–123

Current Microbiology

The Eubacterial Endosymbionts of Whiteflies (Homoptera: Aleyrodoidea) Constitute a Lineage Distinct from the Endosymbionts of Aphids and Mealybugs

Marta A. Clark,[1] Linda Baumann,[1] Mark A. Munson,[1] Paul Baumann,[1] Bruce C. Campbell,[2] James E. Duffus,[3] Lance S. Osborne,[4] and Nancy A. Moran[5]

[1]Department of Microbiology, University of California, Davis, California; [2]U.S. Department of Agriculture, ARS, Albany, California; [3]U.S. Department of Agriculture, ARS, Salinas, California; [4]AREC-Apopka, University of Florida, Apopka, Florida; and [5]Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona, USA

Abstract. Whiteflies (superfamily Aleyrodoidea) contain eubacterial endosymbionts localized within host cells known as mycetocytes. Sequence analysis of the genes for the 16S rRNA of the endosymbionts of *Bemisia tabaci*, *Siphoninus phillyreae*, and *Trialeurodes vaporariorum* indicates that these organisms are closely related and constitute a distinct lineage within the γ-subdivision of the *Proteobacteria*. The endosymbionts of whiteflies are unrelated to the endosymbionts of aphids and mealybugs, which are in two separate lineages.

The insect order Homoptera contains a variety of species that feed on plant sap and are major agricultural pests [2–4, 9, 14, 22]. Some of these insects are dependent on a symbiotic association with procaryotic organisms (endosymbionts) that have not been cultured outside the insect host [4, 9, 15]. Evidence has been presented indicating that the endosymbionts provide essential nutrients to the insect host [9, 15, 24]. The endosymbionts are localized in vesicles within specialized polyploid cells (mycetocytes) that form a structure within the body of the insect host known as the mycetome [9, 14]. The evolutionary relationships of selected endosymbionts have been determined by sequence comparison of the DNA coding for 16S rRNA (rDNA). The results indicate that the endosymbionts of aphids (superfamily Aphidoidea) are members of the γ-3 subdivision of the *Proteobacteria* [18], whereas the endosymbionts of mealybugs (family Pseudococcidae within the superfamily Coccoidea) are members of the β-subdivision [20]. The endosymbiotic associations within these two groups are, therefore, a consequence of separate infections of the two insect lineages by different procaryotes. The endosymbiont of aphids has been given the designation *Buchnera aphidicola* [17], and it has been found to have many of the attributes of free-living eubacteria [16, 19].

Whiteflies (superfamily Aleyrodoidea) share some of the general properties of aphids and mealybugs [3, 7, 11]. All three groups are classified within the suborder Sternorrhyncha, which is believed to be a monophyletic group [13]. Whiteflies also contain mycetocytes harboring procaryotic endosymbionts [2, 26]. *Bemisia tabaci* (sweetpotato whitefly), *Trialeurodes vaporariorum* (greenhouse whitefly), and *Siphoninus phillyreae* (ash whitefly) are important agricultural pests [3]. Recently, in the Southwestern United States, a new strain of *B. tabaci* has emerged that has caused major crop losses [1, 6, 22]. This strain differs in a variety of properties from the strain which it has replaced [1, 22]. The original has been called the "cotton" strain, and the more recent strain the "poinsettia" strain [1]. In the present study we have cloned and sequenced the 16S rDNA from the above three species of whiteflies including both the cotton (C) and poinsettia (P) strains of *B. tabaci*. Our results indicate that the whitefly endosymbionts differ from those of aphids and mealybugs and constitute another lineage within the γ-subdivision of the *Proteobacteria*.

Materials and Methods

B. tabaci C and *B. tabaci* P were both supplied by J.E. Duffus (JD) and L.S. Osborne (LO), and their origins will be indicated by the author's initials, in parentheses, following the strain designation. *S. phillyreae* and *T. vaporariorum* were supplied by B.C.

Address reprint requests to: Dr. Paul Baumann, Department of Microbiology, University of California, Davis, CA 95616, USA.

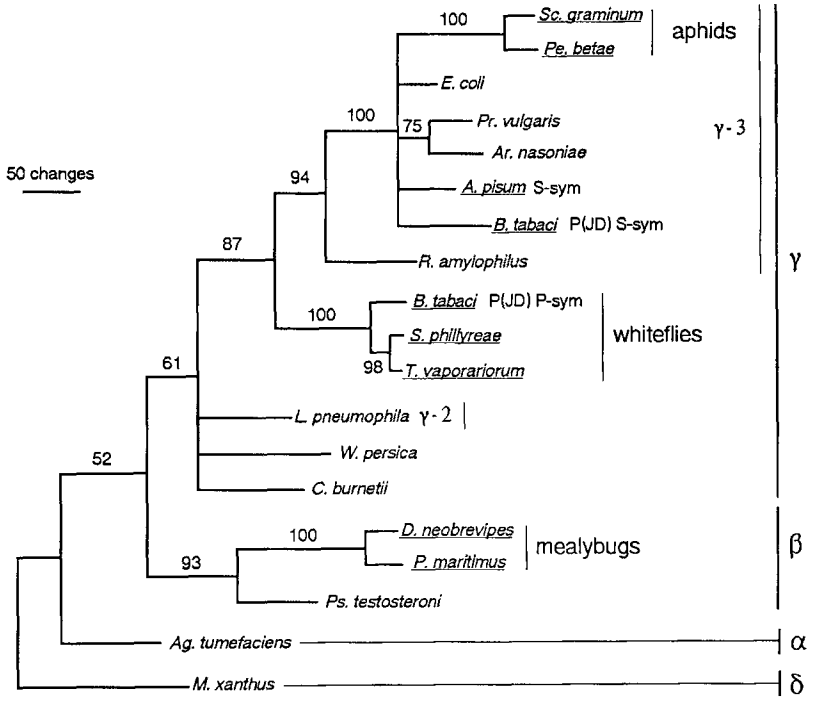

Fig. 1. Phylogenetic tree based on the 16S rDNA of the endosymbionts of three species of whiteflies, representatives of aphid and mealybug endosymbionts, as well as eubacteria representative of the major subdivisions (α, β, γ, δ) of the *Proteobacteria*. Underlined names refer to insect species containing the endosymbionts. P-sym, primary endosymbiont; S-sym, secondary endosymbiont. The following letters designate the genera given in parentheses: A (*Acyrthosiphon*), Ag (*Agrobacterium*), Ar (*Arsenophonus*), B (*Bemisia*), C (*Coxiella*), D (*Dysmicoccus*), E (*Escherichia*), L (*Legionella*), M (*Myxococcus*), P (*Pseudococcus*), Pe (*Pemphigus*), Pr (*Proteus*), Ps (*Pseudomonas*), R (*Ruminobacter*), S (*Siphoninus*), Sc (*Schizaphis*), T (*Trialeurodes*), W (*Wolbachia*). The tree is the majority rule consensus of 100 bootstrap runs; numbers are bootstrap indices of the level of support for individual nodes. The consistency index is 0.474; tree length is 2187.

Table 1. Pairwise distances between bacterial taxa of the γ-subdivision, based on aligned sequence length of 1551 bases

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *Sc. graminum*	—													
2. *Pe. betae*	69	—												
3. *E. coli*	154	153	—											
4. *P. vulgaris*	184	174	93	—										
5. *Ar. nasoniae*	171	170	117	99	—									
6. *A. pisum* S-sym	186	174	81	108	113	—								
7. *B. tabaci* S-sym	178	174	128	136	135	112	—							
8. *R. amylophilus*	225	228	187	193	197	179	213	—						
9. *B. tabaci* P-sym	251	257	258	249	251	246	243	250	—					
10. *S. phillyreae*	235	241	245	229	233	230	225	238	70	—				
11. *T. vaporariorum*	230	236	240	230	232	225	228	242	69	30	—			
12. *L. pneumophila*	235	235	208	210	202	205	218	196	214	203	203	—		
13. *W. persica*	265	262	233	245	248	244	260	225	271	245	249	187	—	
14. *C. burnetii*	269	263	234	249	228	244	253	228	247	229	234	158	208	—

For abbreviations see legend to Fig. 1.

Campbell (BC). The general molecular biology methods used in this study have been described by Sambrook et al. [23]. The methods used for the purification of DNA, amplification of the 16S rDNA by the polymerase chain reaction (PCR), cloning into phagemids, and the sequencing of both DNA strands of the cloned insert have been described in detail [16, 18–20].

Bacteria included in the analysis were chosen to represent established subdivisions of the *Proteobacteria* (α, β, γ, δ), including subgroups of the γ-subdivision [31, 32]. Sequences were taken from Neefs et al. [21]. In addition, other bacterial associates of arthropods for which sequences were available were included. The taxa and the sources of the sequences were as follows: primary endosymbionts of two aphid species [18], secondary endosymbiont of an aphid [27], endosymbionts of two mealybug species [20], intracellular associates of two ticks [28], and an associate of a parasitoid wasp [12].

Nucleotide sequences from the endosymbionts and other bacteria were aligned, and parsimony analysis was carried out with the program "Phylogenetic analysis using parsimony" [25]. To root the tree, *Myxococcus xanthus*, representing the δ-subdivision, was set as outgroup [31]. Gaps were scored as missing data, uninformative sites were excluded from the analysis, and

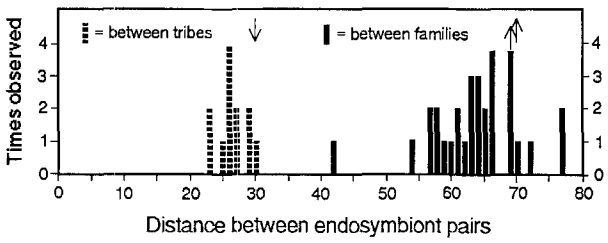

Fig. 2. Distribution of distances (16S rDNA) between pairs of endosymbionts of aphid species in the tribes Aphidini and Macrosiphini and aphid species in the families Aphididae, Drepanosiphidae, Mindaridae, and Pemphigidae [18]. ↓ = distances between *S. phillyreae* and *T. vaporariorum*; ↑ = distances between these two species and *B. tabaci*.

transitions and transversions were weighted equally. There were 587 informative sites and 19 taxa. The dataset was too large for an exhaustive search for the shortest tree, so the heuristic search option was used. The "Bootstrapping" option was used to obtain the majority rule consensus tree consisting of all nodes supported by over half of 100 bootstrap runs.

The nucleotide sequences were deposited in GenBank under accession numbers Z11925–Z11928.

Results and Discussion

Evolutionary relationships of whitefly endosymbionts. The endosymbionts of whiteflies form a cluster of related organisms (Fig. 1) distinct from the endosymbionts of aphids and mealybugs. This cluster is within the γ-subdivision of the *Proteobacteria* between representatives of the γ-2 and γ-3 subgroups. Monophyly of the whitefly endosymbionts is very strongly supported by the sequence data, as indicated by the bootstrap value of 100; there is also strong support for their inclusion in the γ-subdivision. However, it was not possible to assign the endosymbionts to either the γ-3 or γ-2 subgroups on the basis of the phylogenetic analysis or the presence of oligonucleotide signature sequences [32]. In this respect the whitefly endosymbionts resemble *Wolbachia persica* (an intracellular, tick-associated procaryote) [30] and *Coxiella burnetii* (an intracellular, tick-associated procaryote causing Q fever in humans) [29], which are also members of the γ-subdivision but could not be assigned to any of the subgroups [28] (Fig. 1). Examination of Table 1 indicates that *Legionella pneumophila* (a member of the γ-2 subgroup) has the closest distance to the whitefly endosymbionts.

Figure 2 presents the distribution of the observed 16S rDNA distances between endosymbionts of aphid species in different tribes and families [18]. By analogy with aphids, the distances between the endosymbionts of *S. phillyreae* and *T. vaporariorum* (Fig. 2, Table 1) are consistent with the placement of the whitefly hosts into different tribes. The distances between (1) *B. tabaci* P(JD) and (2) *S. phillyreae* and *T. vaporariorum* are consistent with their placement into two families. Currently these whitefly species are assigned to three tribes within a single family [8].

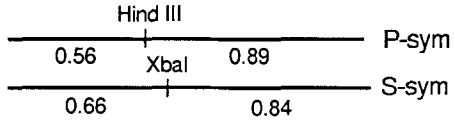

Fig. 3. Position of restriction enzyme sites characteristic of the *B. tabaci* P-sym and the S-sym 16S rDNA. Numbers represent sizes in kilobases.

When the nucleotide sequence of recombinants containing the PCR-amplified 16S rDNA of *B. tabaci* P(JD) was determined, two different sequences were found, indicating the presence of two organisms. One of these organisms was related to the endosymbionts of the two other species of whiteflies (Fig. 1), while the second was related to members of the *Enterobacteriaceae* (γ-3 subgroup). By analogy with the endosymbionts of *Acyrthosiphon pisum*, we have designated these as the primary endosymbiont (P-sym) and the secondary endosymbiont (S-sym), respectively. The S-sym of *B. tabaci* P (JD) is most closely related to the S-sym of *A. pisum* (Fig. 1, Table 1). Other species within this cluster are *Proteus vulgaris* and *Arsenophonus nasoniae* (Fig. 1); the latter is the causative agent of the son-killer trait in a wasp [12]. Recently it has been found that endosymbionts of the coleopterans, *Sitophilus oryzae* and *S. zeamais*, also fall within the γ-3 subgroup [5]. Sequence comparisons indicated that they are distinct from the S-sym of *B. tabaci*.

Comparisons of *B. tabaci* P and C from different sources. We have determined the 16S rDNA sequence of the endosymbiont of *B. tabaci* C(JD) P-sym and have found it to be essentially identical to that of *B. tabaci* P(JD) P-sym. (The two nucleotide differences detected could be owing to PCR error.) An inspection of the sequence of the 16S rDNA of *B. tabaci* P(JD) P-sym and *B. tabaci* P(JD) S-sym indicated the presence of restriction enzyme sites that could differentiate between the PCR-amplified 16S rDNAs of these two organisms. For example, the P-sym 16S rDNA has a *Hin*dIII site (Fig. 3) which, when cut by this enzyme, results in fragments of 0.56 and 0.89 kb. A *Hin*dIII restriction site is absent in the 16S rDNA of the S-sym (Fig. 3). Simi-

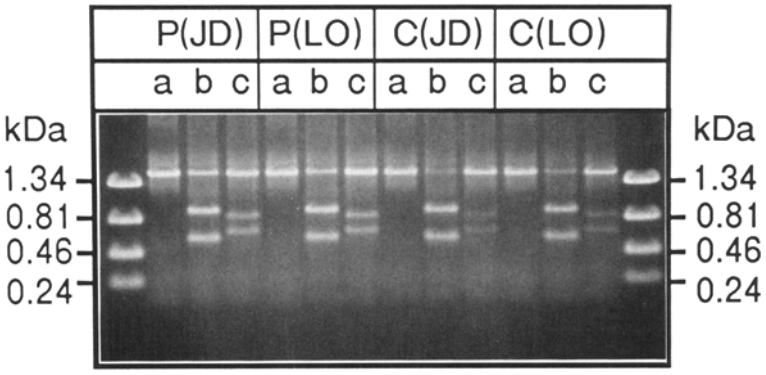

Fig. 4. Agarose gel electrophoresis of PCR-amplified and restriction enzyme-digested 16S rDNA of *B. tabaci*. P, poinsettia strain; C, cotton strain; initials in parentheses indicate investigator who sent the strain. kb, kilobases; a, undigested PCR-product; b, digested with *Hin*dIII; c, digested with *Xba*I.

larly, the S-sym has an *Xba*I restriction site that is absent in the P-sym. Figure 4 gives the results of experiments in which the amplified 16S rDNA of *B. tabaci* P(JD), C(JD), P(LO), and C(LO) was digested with *Hin*dIII and *Xba*I. In all cases, a band of lower intensity remained that corresponded to the 16S rDNA of the endosymbiont that lacked the restriction site as well as two smaller bands corresponding to fragments of the 16S rDNA of the endosymbiont that contained the restriction site. The results with the restriction enzymes *Acc*I, *Ava*I, *Sac*I, and *Sma*I indicate that all of these isolates of *B. tabaci* have a P-sym and a S-sym and that the P-syms are identical to each other on the basis of restriction enzyme analysis, as are also the S-syms.

Conclusions

(1) The whitefly species *B. tabaci*, *S. phillyreae*, and *T. vaporariorum* all have a P-sym that forms a distinct lineage within the gamma-subdivision of the *Proteobacteria*. This result suggests the occurrence of a single infection of a whitefly ancestor with subsequent divergence of the endosymbiont and the host.

(2) The whitefly endosymbionts are distinct from the aphid and mealybug endosymbionts, which are derived from different bacterial lineages.

(3) *B. tabaci* appears to have an S-sym which is a member of the *Enterobacteriaceae* and is related to the S-sym of the aphid *A. pisum*.

(4) The P-syms of *B. tabaci* P and *B. tabaci* C are indistinguishable from each other, as are also the S-syms. This result need not indicate identity of the *B. tabaci* hosts, since 16S rRNA is a highly conserved molecule, and bacterial isolates having identical 16S rRNA sequences may belong to closely related bacterial species readily recognizable on the basis of other genetic or phenotypic properties [10]. One of us (BC) is currently attempting to differentiate *B. tabaci* P and *B. tabaci* C by use of host genetic markers.

ACKNOWLEDGMENTS

This research was supported by grant BSR-9119280 from the National Science Foundation (NM and PB) and the University of California Agricultural Experiment Station (PB). We thank Dr. R. J. Gill for taxonomic advice and the identification of *T. vaporariorum*, and Dr. G. G. Still for ash whiteflies.

Literature Cited

1. Bethke JA, Paine TD, Nuessly, GS (1991) Comparative biology, morphometrics, and development of two populations of *Bemisia tabaci* (Homoptera: Aleyrodidae) on cotton and poinsettia. Ann Entomol Soc Am 84:407–411
2. Buchner P (1965) Endosymbiosis of animals with plant microorganisms. New York: Interscience Publisherse, Inc., pp. 210–338
3. Byrne DN, Bellows TS (1991) Whitefly biology. Annu Rev Entomol 36:431–457
4. Campbell BC (1990) On the role of microbial symbiotes in herbivorous insects. In: Bernays EA (ed) Insect-plant interactions, vol. I. Boca Raton, Fla.: CRC Press, Inc., pp 1–44
5. Campbell BC, Bragg TS, Turner CE (1992) Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) based on analysis of 16S ribosomal DNA. Insect Biochem Mol Biol, in press
6. Cohen S, Duffus JE, Liu HY (1992) A new *Bemisia tabaci* biotype in the Southwestern United States and its role in silverleaf of squash and transmission of lettuce infectious yellows virus. Phytopathology 82:86–90
7. Daly HV, Doyen JT, Ehrlich PR (1978) Introduction to insect biology and diversity. New York: McGraw-Hill Book Co., pp 364–367
8. David BV (1990) Key to tribes of whiteflies (Aleyrodidae: Homoptera) of India. J Insect Sci 3:13–17

9. Douglas AE (1989) Mycetocyte symbiosis in insects. Biol Rev 64:409–434
10. Fox GE, Wisotzkey JD, Jurtshuk P (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 42:166–170
11. Gerling D (ed) (1990) Whiteflies: their bionomics, pest status and management. Newcastle upon Tyne: Athenaeum Press
12. Gherna RL, Werren JH, Weisburg W, Cote R, Woese CR, Mandelco L, Brenner DJ (1991) *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis*. Int J Syst Bacteriol 41:563–565
13. Hennig W (1980) Insect phylogeny (edition with revisionary notes by D Schlee). New York: Wiley and Sons
14. Houk EJ, Griffiths GW (1980) Intracellular symbiotes of the Homoptera. Annu Rev Entomol 25:161–172
15. Ishikawa H (1989) Biochemical and molecular aspects of endosymbiosis in insects. Int Rev Cytol 116:1–45
16. Lai C-Y, Baumann P (1992) Genetic analysis of an aphid endosymbiont DNA fragment homologous to the *rnpA–rpmH–dnaA–dnaN–gyrB* region of eubacteria. Gene 113:175–181
17. Munson MA, Baumann P, Kinsey MG (1991) *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. Int J Syst Bacteriol 41:566–568
18. Munson MA, Baumann P, Clark MA, Baumann L, Moran NA, Voegtlin DJ, Campbell BC (1991) Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. J Bacteriol 173:6321–6324
19. Munson MA, Baumann L, Baumann P (1992) *Buchnera aphidicola*, the endosymbiont of aphids, contains genes for four ribosomal RNA proteins, initiation factor-3, and the α-subunit of RNA polymerase. Curr Microbiol 24:23–29
20. Munson MA, Baumann P, Moran NA (1992) Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera: Pseudococcidae) based on 16S rDNA sequences. Mol Phylogen Evol, in press
21. Neefs J-M, Van de Peer Y, Hendriks L, De Wachter R (1990) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res 18:r2237–r2317
22. Perring TM, Cooper A, Kazmer DJ (1992) Identification of the poinsettia strain of *Bemisia tabaci* (Homoptera: Aleyrodidae) on broccoli by electrophoresis. J Econ Entomol, in press
23. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning; a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press
24. Srivastava PN (1987) Nutritional physiology. In: Minks AK, Harrewijn P (eds) Aphids: their biology, natural enemies, and control, vol. 2A. Amsterdam: Elsevier Biomedical Press, pp 99–121
25. Swofford DL (1991) PAUP: phylogenetic analysis using parsimony. MacIntosh version 3.0r+3. Champaign, Ill.: Illinois Natural History Survey
26. Tremblay E (1959) Osservazioni sulla simbiosi endocellulare di alcuni Aleyrodidae (*Bemisia tabaci* Gennad., *Aleurolobus olivinus* Silv., *Trialeurodes vaporariorum* West.). Boll Lab Entomol Agric "F. Silvestry" Portici 27:210–246
27. Unterman BM, Baumann P, McLean DL (1989) Pea aphid symbiont relationships established by analysis of 16S rRNAs. J Bacteriol 171:2970–2974
28. Weisburg WG, Dobson ME, Samuel JE, Dasch GA, Mallavia LP, Baca O, Mandelco L, Sechrest JE, Weiss E, Woese CR (1989) Phylogenetic diversity of the *Rickettsiae*. J Bacteriol 171:4202–4206
29. Weiss E, Moulder JW (1984) Coxiella. In: Krieg NR, Holt JG (eds) Bergey's manual of determinative bacteriology, vol. 1. Baltimore: Williams & Wilkins, pp 701–704
30. Weiss E, Dasch GA, Chang K-P (1984) Wolbachia. In Krieg NR, Holt JG (eds) Bergey's manual of determinative bacteriology, vol. 1. Baltimore: Williams & Wilkins, pp 711–713
31. Woese, CR (1987) Bacterial evolution. Microbiol Rev 51:221–271
32. Woese CR, Weisburg WG, Hahn CM, Paster BJ, Zablen LB, Lewis BJ, Macke TJ, Ludwig W, Stackebrandt E (1985) The phylogeny of purple bacteria: the gamma subdivision. Syst Appl Microbiol 6:25–33